Characterization of Poly(A)-Protein Complexes Isolated from Free and Membrane-Bound Polyribosomes of Ehrlich Ascites Tumor Cells

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CHARACTERIZATION OF POLY(A)-PROTEIN COMPLEXES ISOLATED FROM FREE AND MEMBRANE-BOUND POLYRIBOSOMES OF EHRLICH ASCITES TUMOR CELLS

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Abstract. Proteins present in messenger ribonucleoprotein particles were labeled with [^{35}S]-methionine in Ehrlich ascites tumor cells in which synthesis of new ribosomes was inhibited. Poly(A)-protein complexes were isolated from free and membrane-bound polyribosomes by sucrose gradient centrifugation and affinity chromatography on oligo(dT)-cellulose. Both classes of Poly(A)-protein particles contain a poly(A) chain of about 70 adenyl residues and a protein with a molecular weight of 76000 attached to it.

I. INTRODUCTION

Eukaryotic messenger RNA is usually associated with proteins in the form of a messenger ribonucleoprotein comples (mRNP) [1]. At least one of these proteins, a 78000 daltons protein, has been found to be tightly associated with the poly(A)-segment of messenger RNA from free polyribosomes [2]. No comparative data on the protein composition of poly(A)-segments of free and membrane-bound messenger RNA are available. This would be of particular interest, since recent studies have indicated that the poly(A)-segment of the messenger RNA of membrane-bound polyribosomes is attached to membrane structures in HeLa cells [3] and human diploid fibroblasts [4]. The possibility that a protein associated with the poly(A)-segment could be responsible for the interaction between poly(A) and the membrane was suggested [3]. We have found that also in Ehrlich ascites cells interaction between poly(A) and cytoplasmic membranes occurs (W.J. van Venrooij, unpublished results). Therefore, we compared the protein composition of poly(A)-protein particles isolated from free polyribosomes and those which were bound to cytoplasmic membranes. We labeled the proteins present on the mRNA while synthesis of new ribosomes was inhibited. This method allows a rather specific labeling of the proteins tightly associated with mRNA.

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II. MATERIALS AND METHODS

Ethidium bromide and actinomycin D were obtained from Sigma. [^{35}S]-methionine (specific activity 140 Ci x mmole^{-1}) and [^{3}H]-adenosine (specific activity 6.4 Ci x mmole^{-1}) were supplied by the Radiochemical Centre, Amersham. Oligo(dT)-cellulose (T_2) was purchased from Collaborative Research, Boston, Massachusetts.

All glassware was sterilized and solutions were autoclaved or treated with diethylpyrocarbonate before use.

Cell growth and labeling

Ehrlich ascites tumor cells were grown in suspension culture as described earlier [5, 6]. For labeling of mRNA the medium was replaced by fresh medium (2 x 10^6 cells x ml^{-1}). After 30 min actinomycin D and ethidium bromide (0.04 μg x ml^{-1} and 1 μg x ml^{-1}, respectively) and after another 30 min [^{3}H]-adenosine (2 μCi x ml^{-1}) were added. Cells were harvested after a labeling period of 3 h.

For labeling of mRNP proteins cells were incubated in fresh medium without methionine (4 x 10^6 cells ml^{-1}). Actinomycin D and ethidium bromide were added 30 min prior to labeling with [^{35}S]-methionine (4.5 μCi x ml^{-1}). After a labeling period of 4 h the medium was replaced by fresh, complete medium containing actinomycin D and ethidium bromide. Cell growth was continued for an additional 4 h to ensure reformation of polyribosomes. In some experiments [^{3}H]-adenosine was added during this period to obtain double-labeled ribonucleoprotein particles.

Cell fractionation and isolation of polyribosomes

Cell homogenization and fractionation were performed as described earlier [6]. The 20000 x g pellet (nuclei, mitochondria and membranes) was suspended in 8 ml buffer 1 (50 mM KCl, 10 mM TRIS-HCl, pH 7.4, 5 mM $MgCl_2$, and 6 mM β-mercaptoethanol), and 0.1 volume of a mixture of 5% sodium deoxycholate and 3% Nonidet-P40 was added to dissolve the membranes. After centrifugation at 2000 x g for 5 min to remove the nuclei, the suspension was layered on 2 ml 2 M sucrose in buffer 1. Centrifugation at 164000 x g for 16 h yielded a pellet of membrane-derived polyribosomes. Similarly, centrifugation of the 20000 x g supernatant over 2 M sucrose in buffer 1 yielded a pellet of free polyribosomes.

Salt wash and RNase treatment of polyribosomes

The polyribosome pellets were suspended in 4 ml buffer 2 (500 mM KCl, 50 mM TRIS-HCl, pH 7.4, 5 mM $MgCl_2$, and 6 mM β-mercaptoethanol) and centrifuged over 2 ml 1 M sucrose in buffer 2 for 4 h at 164000 x g. The upper 4 ml of the gradient were kept for analysis of the proteins which were removed from the polysomes during the salt wash. The pellets of high salt-washed polyribosomes were suspended in 0.5 ml buffer 3 (50 mM KCl, 50 mM TRIS-HCl, pH 7.4), and incubated with a mixture of RNase A and RNase T_1 (final concentrations 1 μg x ml^{-1} and 1 U x ml^{-1}, respectively) at 30 °C for 30 min. In case the material had to be used for further purification on oligo(dT)-cellulose incubation with ribonuclease was carried out in buffer 4 (200 mM NaCl, 50 mM TRIS-HCl, pH 7.8, and 10 mM EDTA) containing 0.2% Nonidet-P40. A small precipitate that arose during RNase treatment was centrifuged off at low speed and the clear suspension was used for further fractionation.

Polyacrylamide gel electrophoresis of proteins

Polyacrylamide gel electrophoresis in 13% polyacrylamide slab gels containing 0.1% sodium dodecylsulfate was performed as described earlier [7]. Protein samples were precipitated by adding 50 μg αA_2 crystallin as a carrier and 0.2 volume 50% trichloroacetic acid. The precipitates were washed with acetone, air-dried, dissolved in 20 μl 2% sodium dodecylsulfate and 5% β-mercaptoethanol, and heated for 1 min at 100 °C. Slab gels were treated with dimethylsulphoxide-2,5-diphenyloxazol [8], and dried down on filter paper before scintillation autoradiography.

Oligo(dT)-cellulose chromatography and length determination of poly(A)

Protein-free poly(A) tracks were purified on oligo(dT)-cellulose in the presence of sodium dodecylsulfate [9]. Determination of the length was done by alkaline hydrolysis of lyophilized poly(A) chains as described by Mendecki *et al.* [10]. Poly(A)-protein complexes were purified on oligo(dT)-cellulose as described by Lindberg and Sundquist [11] for mRNA-protein complexes.

III. RESULTS AND DISCUSSION

For proper comparison of the poly(A)-segments and their protein moiety of mRNP present in free and membrane-bound polyribosomes it is necessary to obtain a good separation between these two classes of polyribosomes. This was achieved by isolation of the membrane fraction directly after cell homogenization (in the presence of 0.25 M sucrose to minimize nuclear leakage) by a centrifugation for 10 min at 20000 x g [6]. It has been shown earlier [6] that the pellet containing nuclei, mitochondria and membranes, does not contain significant amounts of free polyribosomes. Rehomogenization of the pellet with a loose fitting Dounce homogenizer does not release (poly)ribosomes indicating that the polyribosomes in this fraction are tightly associated with the membranes. Treatment of the pellet with a mixture of sodium deoxycholate and Nonidet-P40 releases undegraded membrane-bound polyribosomes [6] and intact nuclei. The polyribosomes of the 20000 x g supernatant and of the solubilized 20000 x g pellet were salt-washed as described in the methods section. This salt wash procedure was found to be essential for the removal of loosely bound and non-specifically adsorbed proteins from the polyribosomes. About 30% of the [^{35}S]-radioactivity present in unwashed polyribosome pellets is removed by the high salt wash employed.

To isolate the poly(A)-protein complexes we treated the salt-washed polyribosomes with a mixture of ribonuclease A and ribonuclease T_1 (1 μg x ml^{-1} and 1 U x ml^{-1}, respectively). During this treatment a small precipitate was formed which was analyzed on sodium dodecylsulfate containing polyacrylamide gels.

The ribonuclease resistant material was used in two types of experiments. In some experiments it was used for purification of the poly(A) chains on oligo(dT)-cellulose [9] and determination of the average length of these chains as described by Mendecki *et al.* [10]. In other experiments the ribonuclease resistant material was further purified for detection of the proteins present on the poly(A). No significant differences could be found between the length of the poly(A) tracks from free and membrane-bound polyribosomes. Both classes of poly(A) tracks contain about 70 adenyl residues. Similar values were found when the ribonuclease treatment was performed

without RNase T_1. Thus, the polyribosomal poly(A) tracks in Ehrlich ascites tumor cells are of about the same length as those found in rabbit reticulocytes [12], but shorter than in mouse sarcoma 180 ascites cells [12] and in HeLa cells [13]. In an isokinetic sucrose gradient the poly(A) tracks of both classes of polyribosomes sediment at around 4S [6].

When the ribonuclease treated polyribosomes are applied onto an isokinetic sucrose gradient and centrifuged, the poly(A)-protein complexes sediment at about 12S (Figure 1). That most

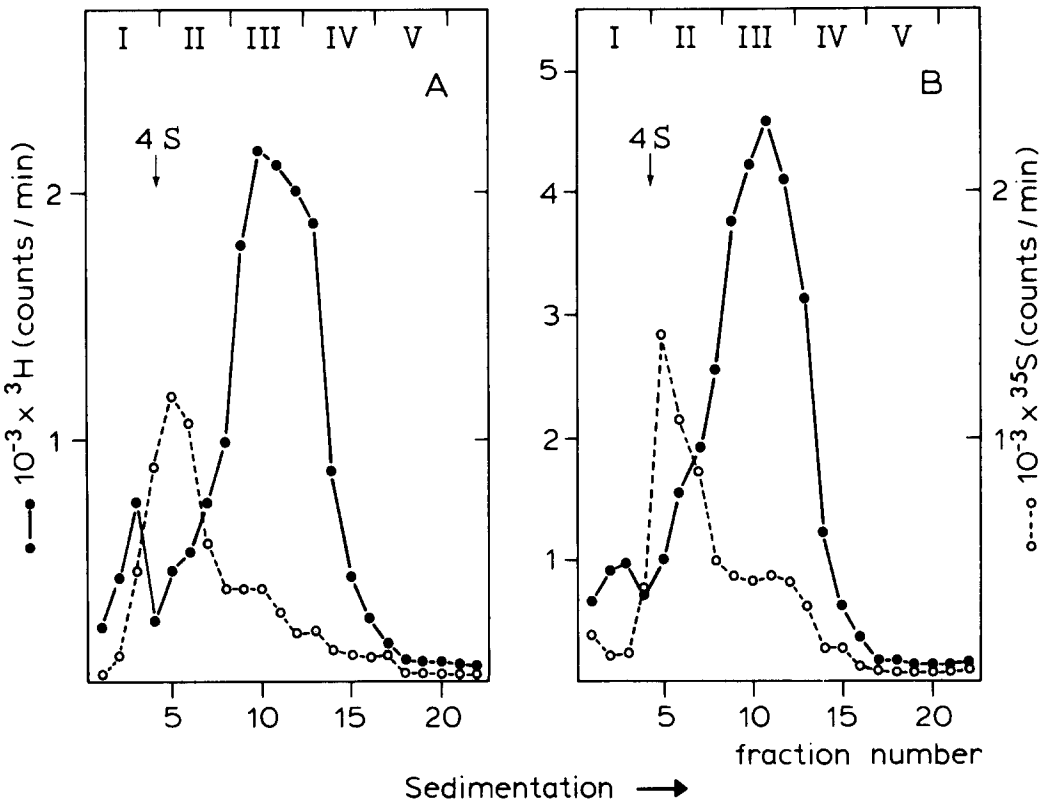

Fig. 1. Sedimentation characteristics in sucrose gradients of poly(A)-protein complexes obtained by ribonuclease treatment of free and membrane-bound polyribosomes.
High salt-washed polyribosomes, double-labeled with [^{3}H]-adenosine and [^{35}S]-methionine, were isolated and digested with a mixture of RNase A and RNase T_1 as described in the methods section. 0.5 ml fractions of the digested material was layered on top of 15–30% isokinetic sucrose gradients in 50 mM TRIS-HCl, pH 7.4. After centrifugation for 24 h at 200000 x g in a IEC-SB283 type rotor fractions were collected and tested for cold trichloroacetic-acid-precipitable radioactivity. The arrows indicate the position of E. coli tRNA which was centrifuged in a different tube. The Roman numerals indicate the fractions collected for analysis of protein composition (see Figure 2).
(A) material from digested free polyribosomes;
(B) material from digested membrane-bound polyribosomes.
●—●—● [^{3}H]-radioactivity; o- -o- -o [^{35}S]-radioactivity.

of the [^{3}H]-label detected in these peaks is present in poly(A) tracks was concluded from the fact that all RNase resistant TCA-precipitable [^{3}H] radioactivity in the gradient adsorbs onto oligo(dT)-cellulose columns (compare Figure 3). The labeled proteins present in the different fractions of such gradients (Figure 1: I, II, III, IV, and V) were analyzed on sodium

dodecylsulfate containing polyacrylamide gels (Figure 2). The most obvious feature is the high degree of similarity between the protein patterns of free and membrane-bound poly(A)-protein complexes. Further, in that region of the gradients were the poly(A)-peak is present (III and IV) a protein with a molecular weight of about 76000 is dominant (Figures 2A: III and 2B: III) suggesting that the poly(A) tracks of both classes are associated with this protein.

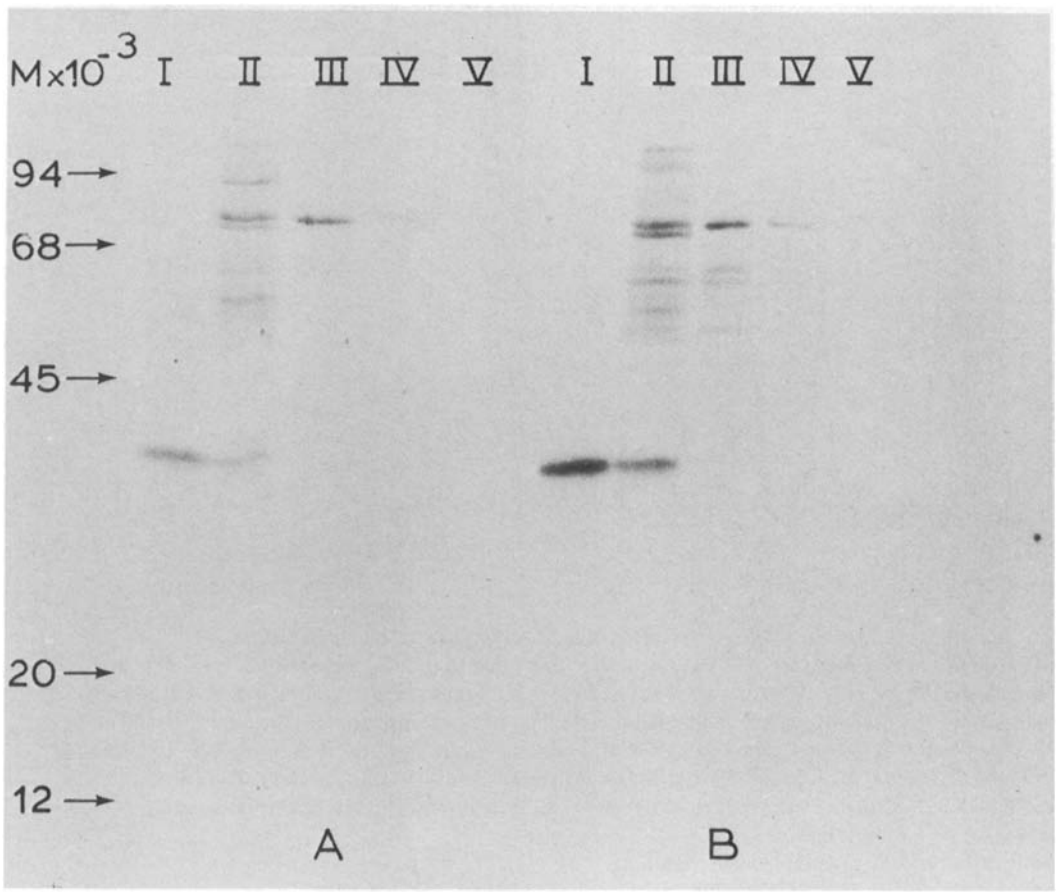

Fig. 2. Autoradiograph of a polyacrylamide gel showing patterns of proteins present in the different fractions of sucrose gradients from the type shown in Figure 1. Ribonuclease-digested polyribosomes were sedimented in sucrode gradients as described in the legend of Figure 1. The sucrose gradient fractions pooled (A: I-V and B: I-V) were analyzed on 13% polyacrylamide gels. Scintillation autoradiography was performed as described in the methods section.

A: I-V: fractions corresponding with sucrose gradient fractions: A: I-V of Figure 1, material from ribonuclease-digested free polyribosomes;

B: I-V: fractions corresponding with sucrose gradient fractions B: I-V of Figure 1, material from ribonuclease-digested membrane-bound polyribosomes.

As marker proteins were used: phosphorylase a (94000), bovine serum albumin (68000), ovalbumin (45000), αA_2 crystallin (20000) and cytochrome c (12000).

Additional evidence for such an association could be obtained after purification of poly(A)-protein complexes on oligo(dT)-cellulose columns as described by Lindberg and Sundquist [11]. Figure 3 shows the elution profile of the ribonuclease treated free polyribosomes when applied

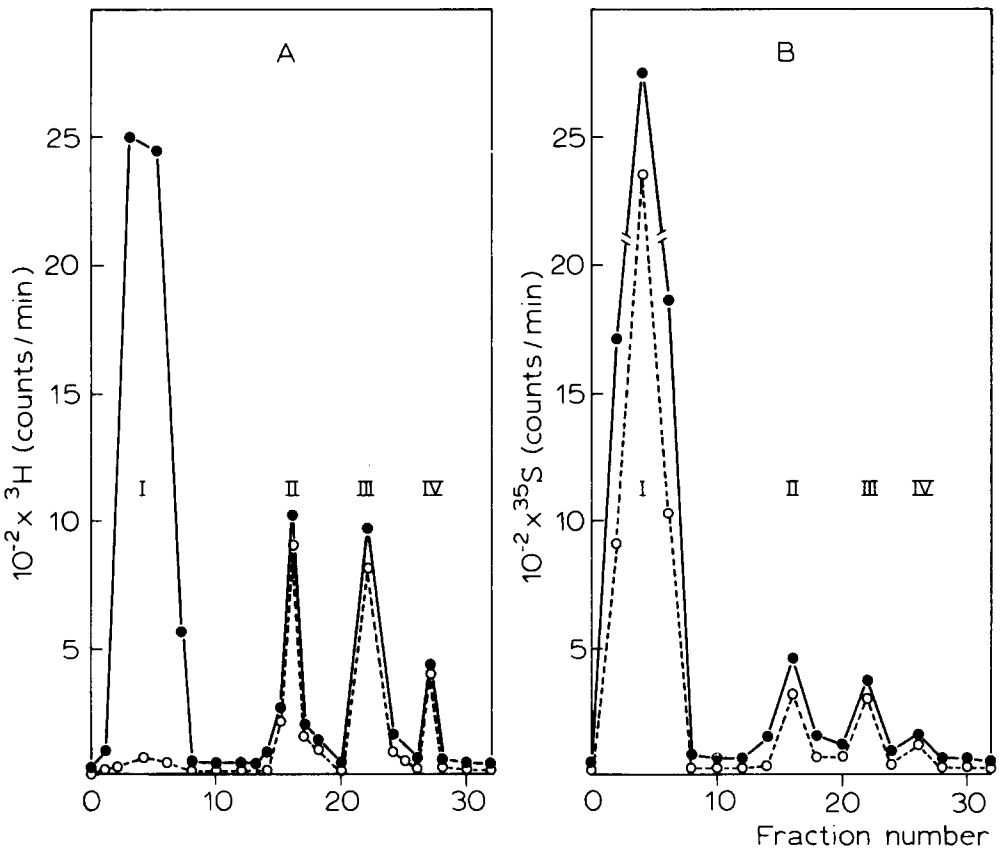

Fig. 3. Oligo(dT)-cellulose chromatography of ribonuclease-digested free polyribosomes. Oligo(dT)-cellulose chromatography was performed as described by Lindberg and Sundquist [11]. Briefly, the ribonuclease-treated polyribosomes were applied in buffer 4, containing 0.2% Nonidet-P40, and the columns were washed free of unadsorbed material with the same buffer (peak I). Elution was performed with 25% formamide in buffer 4 followed by 50% formamide in buffer 4 and 50% formamide in 500 mM NaCl, 50 mM TRIS-HCl, pH 7.8, and 10 mM EDTA (peak II, III and IV, respectively). The elution profile was determined by measuring total radioactivity (●—●) and trichloroacetic-acid-precipitable radioactivity (o- -o) of 3 drop fractions.
(A) fractionation profile of [^{3}H]-labeled material;
(B) fractionation profile of [^{35}S]-labeled material
Note the absence of trichloroacetic-acid-insoluble radioactivity in the non-bound fraction when [^{3}H]-labeled material is fractionated (A).

directly on an oligo(dT)-cellulose column. The elution profile of the membrane-bound polyribosomes was identical and is, therefore, not shown. The elution profile demonstrates that a nearly complete adsorption of ribonuclease-resistant material by the column was obtained. Almost no acid-precipitable [^{3}H]-radioactivity is found in the non-bound fraction (Figure 3A). The ribonuclease-resistant material present in eluted peaks I, II, and III (Figure 3) was further analyzed by sucrose gradient centrifugation and polyacrylamide gel electrophoresis of the proteins. Eluted peak IV was not further analyzed.

Figure 4 shows the pattern of the proteins present in the high salt wash, in the precipitate formed during ribonuclease treatment and in the fractions from the oligo(dT)-cellulose column of free and membrane-bound polyribosomes. In the fractions eluted by formamide the protein

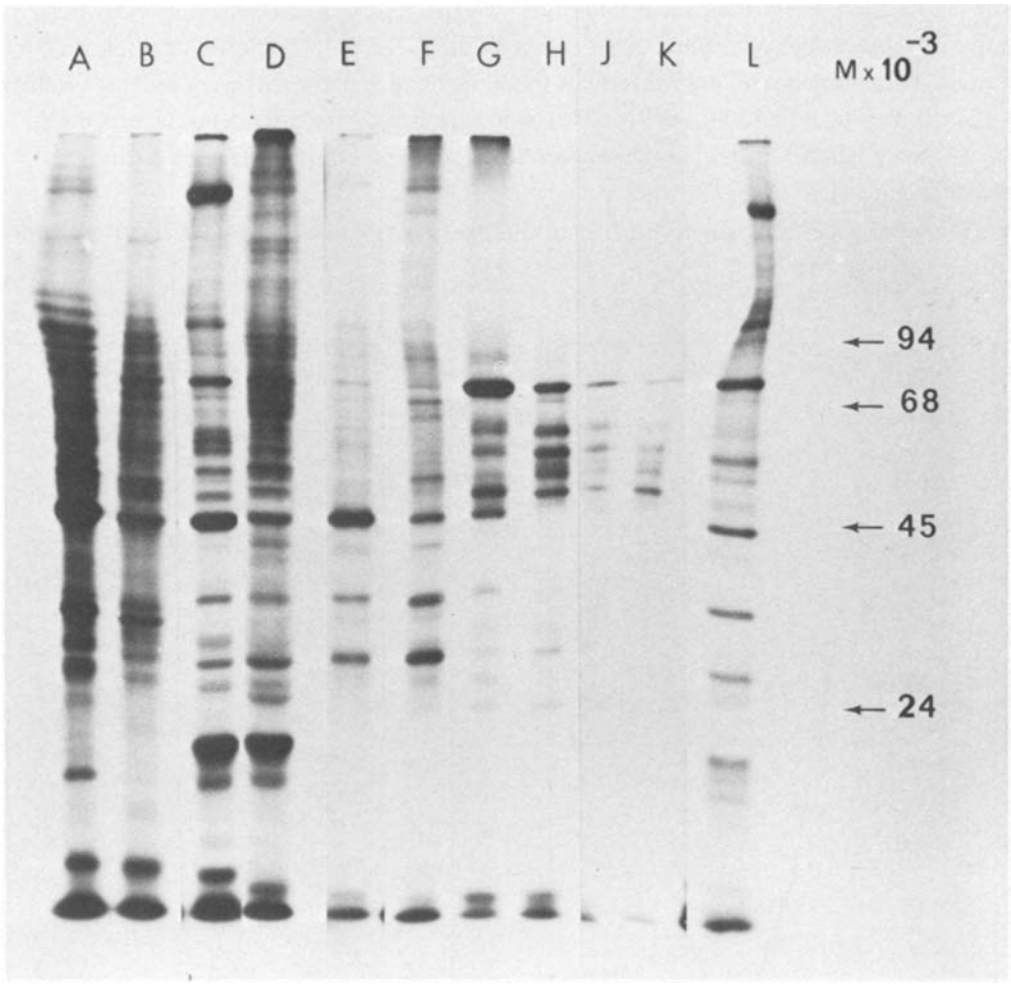

Fig. 4. Autoradiograph of a polyacrylamide gel showing patterns of proteins extracted or released from polyribosomes and poly(A)-protein complexes.
(A) proteins removed from free polyribosomes by the 0.5 M KCl wash;
(B) proteins removed from membrane-bound polyribosomes by the 0.5 M KCl wash;
(C) proteins precipitated during ribonuclease treatment of free polyribosomes;
(D) as C for membrane-bound polyribosomes;
(E-K) protein composition of fractions obtained by oligo(dT)-cellulose chromatography;
(E) non-bound fraction of ribonuclease-digested free polyribosomes;
(F) as E for membrane-bound polyribosomes;
(G) adsorbed (poly(A) containing) material derived from free polyribosomes eluted in 25% formamide (peak II in Figure 3);
(H) as G for membrane-bound polyribosomes;
(J) adsorbed material derived from free polyribosomes eluted by 50% formamide (peak III in Figure 3);
(K) as J for membrane-bound polyribosomes;
(L) labeled proteins present in high salt-washed total polyribosomes not treated with ribonuclease.
As marker proteins were used: phosphorylase a (94000), bovine serum albumin (68000), ovalbumin (45000), and chymotrypsinogen A (24000).

with a molecular weight of 76000, described earlier by Blobel [2] which has also been found in the sucrose gradient fractions containing poly(A) (compare Figures 2A: III and 2B: III), is present next to some other proteins with molecular weights between 62000 and 46000. The quantitative differences between the protein composition of the poly(A)-protein complexes from free and membrane-bound polyribosomes found in some experiments were not consistently seen. Further it may be noted that the 76000 protein attached to the poly(A) track seems also present as a heavily labeled protein in salt-washed total polyribosomes not treated with ribonuclease (Figure 4L).

Figure 5 shows the sucrose gradient porfiles of the ribonuclease-resistant material eluted from

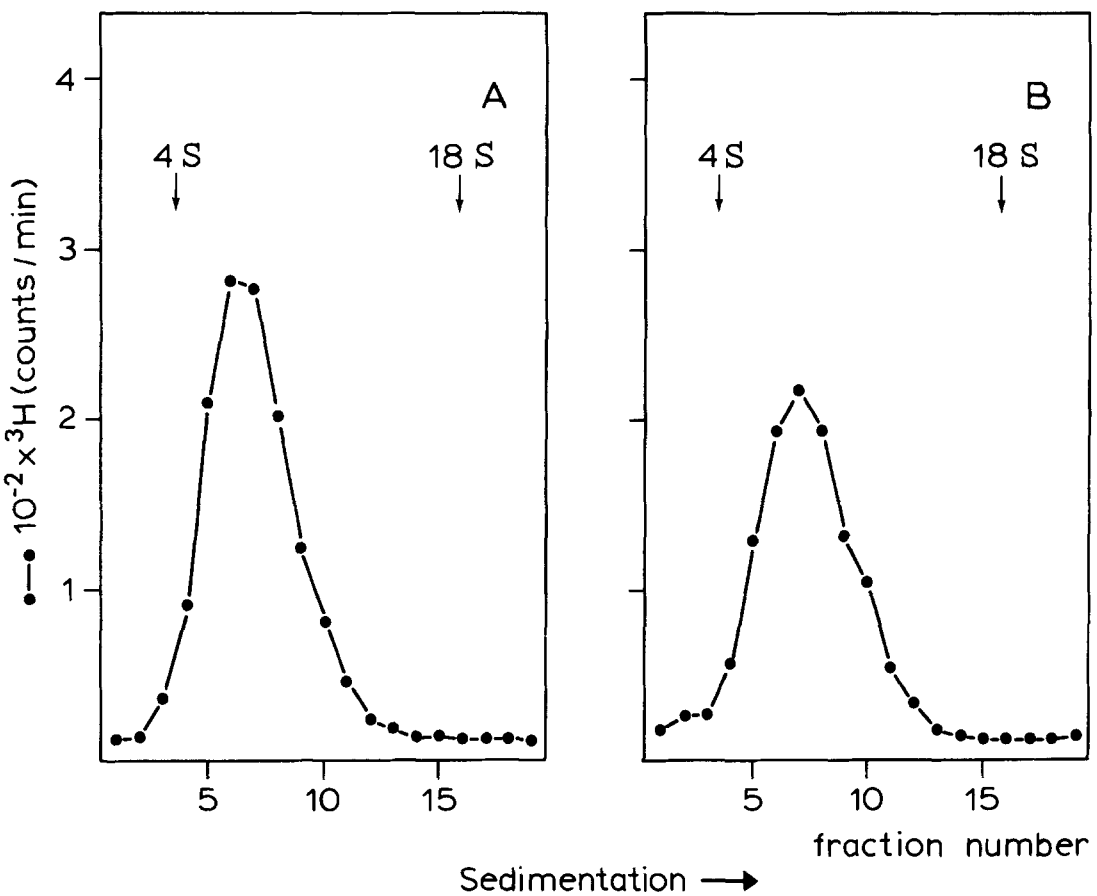

Fig. 5. Sedimentation characteristics in sucrose gradients of poly(A) - protein complexes eluted from the oligo(dT)-cellulose column.
The formamide eluates (peak II and III of Figure 3) were diluted with 2 volumes 50 mM TRIS-HCl, pH 7.4, and centrifuged on sucrose gradients as described in Figure 1.
(A) oligo(dT)-eluate from digested free polyribosomes;
(B) oligo(dT)-eluate from digested membrane-bound polyribosomes.
Sedimentation values of the poly(A)-protein complexes were estimated from the position of 4S *E. coli* tRNA and 18S reticulocyte rRNA, which were co-centrifuged in a different tube.
$\bullet$—$\bullet$ trichloroacetic-acid-precipitable radioactivity.

the oligo(dT)-cellulose column. In this type of experiment the material of peaks II and III (Figure 3) was pooled, because no significant differences in protein composition and sedimentation behavior were found. The sedimentation value of the eluted material was about 8S for both fractions. This is larger than described for protein-free poly(A) tracks (4S, cf. ref. [6]), but smaller than the value obtained when the ribonuclease-resistant material was applied directly onto a sucrose gradient before the oligo(dT)-cellulose procedure (Figure 1). A modification of the structure of poly(A)-protein complexes by the formamide is a possible explanation for this difference.

Our present results demonstrate that the protein composition of poly(A)-protein complexes obtained from free and membrane-bound polyribosomes of Ehrlich ascites tumor cells is about identical. Although this finding does not exclude a possible regulatory or discriminating role of this protein in the binding of mRNA to cytoplasmic membranes, it certainly does not support such a hypothesis. Further work on the identification of messenger RNA specific proteins is in progress now.

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